Novel Bacteriorhodopsin Analogues Based on Azo Chromophores

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Received December 28, 1995[⊗]

Abstract: Bacteriorhodopsin analogues BR−**I**, BR−**II** and BR−**III** containing azo chromophores 4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]azo]benzaldehyde, 3-[4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]azo]phenyl-1]prop-2-enal and 3-[4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]azo]phenyl-1]-2-methylprop-2-enal, respectively, were prepared and characterized by UV−vis spectroscopy, opsin shift, competitive binding with retinal, fluorescence spectroscopy, light-induced pH change, and flash photolysis. BR−**I**, BR−**II**, and BR−**III** had UV−vis absorption maxima at 458, 597, and 485 nm and opsin shifts of −329, 3091, and 43 cm^{-1}, respectively. Competitive binding studies showed that the azo chromophores could not be easily displaced by retinal. Quenching of protein fluorescence by the azo chromophores indicated intimate interactions occurring between the respective azo chromophores and the protein bound residues. The proteins were also found to show functional characteristics (light-induced pH change and flash photolysis profiles) different from those of native bacteriorhodopsin. The results are discussed in terms of the nature of interaction between the azo chromophore and the surrounding protein.

Introduction

Bacteriorhodopsin (BR), the membrane bound protein in the purple membrane (PM) of *Halobacterium halobium* contains the all-*trans*/13-*cis*-retinylidene Schiff base chromophore and functions as a light-driven proton pump.[1] In recent years much effort has been directed toward understanding the unique spectral and photochemical properties of BR. In this context, a large number of synthetic chromophore analogues have been incorporated into the binding site of BR, and the observation of the resulting opsin shifts and/or photochemical properties has been analyzed in terms of chromophore−protein interactions.[2,3] More recently, research on the development of BR variants has also gained momentum due to the possibility of producing proteins of enhanced performance for applications in molecular electronics.[3] BR analogues having chromophores which alter the protein's absorption characteristics while maintaining the photochemical activity and those with chromophores which slow the decay of the M intermediate have been developed.[3]

We report herein the preparation and characterization of BR analogues based on azobenzene chromophores which show unique spectral and photochemical properties.

Experimental Section

Materials and Methods. all-*trans*-Retinal (Sigma), *trans*-cinnamaldehyde, *trans*-α-methylcinnamaldehyde, and *N*,*N*-dimethylaniline (Aldrich) were used as received. *n*-Butylamine (Aldrich) was stored over potassium hydroxide pellets and freshly distilled under nitrogen before use. All other chemicals were from Sisco Research Laboratories (India). Purple membrane fragments were isolated and purified from *H. halobium* (R_1M_1) using the procedure described by Oesterhelt and Stoeckenius.[4] Bacterioopsin was obtained by bleaching the purple membrane according to the procedure described earlier.[5] The apomembrane was pelleted and washed three times with water to remove excess hydroxylamine and retinal oxime. The apomembranes were suspended in Tris·HCl buffer (1.0×10^{-2} M). The pH of the buffer was adjusted by addition of dilute HCl or dilute NaOH solution as required. All pH measurements were done on a Radiometer PHM84 research pH meter equipped with GK 2401 C combination electrodes. UV−vis measurements were recorded on a Hitachi U-2000 spectrophotometer. IR spectra were recorded on a Nicolet Impact 400 FTIR spectrometer. NMR spectra were recorded on a Varian VXR-300MHz spectrometer. Centrifugations were done on a Beckman L-8-55M preparative ultracentrifuge using a 45 Ti rotor. Flash photolysis profiles were obtained on an Applied Photophysics flash kinetic spectrometer (model 858), the output of which was fed to a Philips PM3350 digital storage oscilloscope which recorded the flash photolysis traces. The excitation flash was provided by a U-tube flash lamp (40 J, 900 V, <100 μs) with a range from 400 to 700 nm mounted adjacent to the sample holder, and the detection system consisted of a tungsten−halogen lamp (12 V, 20 W) and a photomultiplier tube. Fluorescence spectra were recorded on a Spex Spectrofluorolog spectrofluorimeter with a 450 W xenon arc lamp as the light source. Data were analyzed on a DM-1B data processor interfaced with the spectrofluorimeter. Molecular mechanics calculations were carried out using the PCMODEL molecular modeling software (4th ed., copyright 1990 Serena Software, Box 3076, Bloomington, IN 47402-3076) on an 80486 IBMPC.

Synthesis of Azo Chromophores I, II, and III. The synthetic procedure of the compounds 4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]azo]-benzaldehyde (**I**), 3-[4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]azo]phenyl-1]prop-2-enal (**II**), and 3-[4-[[4′-(*N*,*N*-dimethylamino)phenyl-1′]-azo]phenyl-1]-2-methylprop-2-enal (**III**) is shown in Scheme 1. 4-Ami-

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

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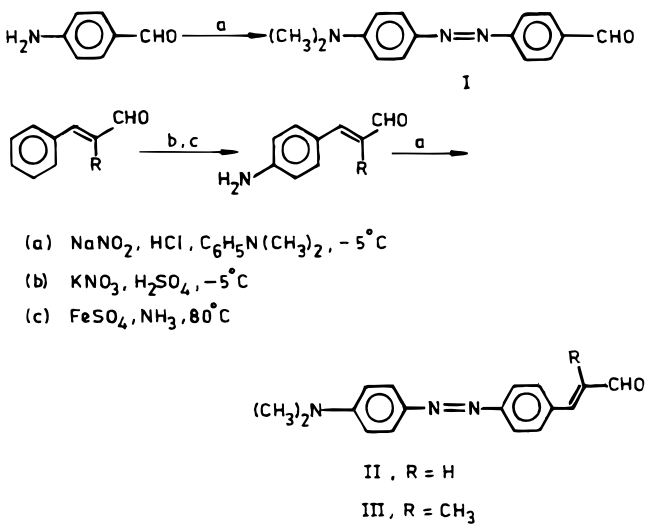
S0002-7863(95)04286-7 CCC: $12.00

Scheme 1



nobenzaldehyde was synthesized following literature procedure.[6] Nitration of *trans*-cinnamaldehyde and *trans*-α-methylcinnamaldehyde was done as reported elsewhere.[7] The reduction of the nitro compounds to the corresponding amines was done following known procedures.[8] Coupling of *N*,*N*-dimethylaniline with the corresponding amines was done using a slight modification of known procedures.[9] This was done to avoid self-condensation of the compounds having both the amino and aldehyde groups into polymeric Schiff bases. In a typical procedure, concentrated HCl (0.4 mL) was dissolved in 20 mL of water and cooled to 0 °C with stirring. A solution of amino compound (0.5 mmol) in acetone was added dropwise to the cooled solution over a period of 30 min with constant stirring. A precooled solution of $NaNO_2$ (37.0 mg, 0.53 mmol) in 2 mL of water was added dropwise to the stirred suspension. A pinch of urea was added to the vigorously stirred solution to destroy any excess nitrous acid remaining in solution. To this solution was added dropwise a solution of *N*,*N*-dimethylaniline (63.0 μL, 0.5 mmol) in 3 mL of glacial acetic acid with stirring. An opaque yellow-red suspension resulted. This solution was vigorously stirred for another 10 min, after which a saturated solution of sodium acetate was added. Bright orange-yellow (for compound **I**) or orange-red (for compounds **II** and **III**) flakes of the azo compounds appeared. The crystalline solids were filtered and dissolved in acetone. The compounds were purified by column chromatography over a silica gel column and characterized by NMR, UV−vis, and IR spectroscopy. The characterization data for the three azo compounds are as follows (s = singlet, d = doublet, m = multiplet; ϵ values are in L mol^{-1} cm^{-1}).

4-[[4′-(*N*,*N*-Dimethylamino)phenyl-1′]azo]benzaldehyde (I). mp: 164−165 °C (uncorrected). Yield: 92%. NMR ($CDCl_3$, δ, ppm): 3.12 (6H, s, NMe_2), 6.75 (2H, d, J = 9.0 Hz, aromatic), 7.94 (6H, m, aromatic), 10.05 (1H, s, C*H*O). UV−vis (MeOH, λ_{max}, nm): 229 (ϵ = 14 500), 284 (ϵ = 18 200), 449 (ϵ = 22 500). IR ($CHCl_3$, cm^{-1}): 3010, 1690, 1590, 1515, 1425, 1360.

3-[4-[[4′-(*N*,*N*-Dimethylamino)phenyl-1′]azo]phenyl-1]prop-2-enal (II). mp: 204−205 °C (uncorrected). Yield: 97%. NMR ($CDCl_3$, δ, ppm) 3.11 (6H, s, NMe_2), 6.76 (3H, m, aromatic + olefinic), 7.53 (1H, d, J = 16.0 Hz, Ar-CH, olefinic), 7.68 (2H, d, J = 8.4 Hz, aromatic), 7.84 (4H, m, aromatic), 9.72 (1H, d, J = 7.7 Hz, C*H*O). UV−vis (MeOH, λ_{max}, nm): 253 (ϵ = 5382), 313 (ϵ = 10 928), 461 (ϵ = 18 836). IR ($CHCl_3$, cm^{-1}): 3010, 1678, 1602, 1522, 1475, 1426, 1365.

3-[4-[[4′-(*N*,*N*-Dimethylamino)phenyl-1′]azo]phenyl-1]-2-methyl-prop-2-enal (III). mp: 150−151 °C (uncorrected). Yield: 94%. NMR ($CDCl_3$, δ, ppm): 2.14 (3H, s, Me), 3.11 (6H, s, NMe_2), 6.77 (2H, d, J = 9.0 Hz, aromatic), 7.30 (1H, s, Ar-CH, olefinic), 7.66 (2H, d, J = 8.7 Hz, aromatic), 7.90 (4H, m, aromatic), 9.61 (1H, s, C*H*O). UV−vis (MeOH, λ_{max}, nm): 251 (ϵ = 7422), 310 (ϵ = 8698), 450 (ϵ = 17 460). IR ($CHCl_3$, cm^{-1}) 3010, 1676, 1603, 1521, 1478, 1425, 1366.

Formation and Protonation of the *n*-Butylamine Schiff Bases of Compounds I−III. Azo chromophore **I** (2.8×10^{-3} M) was taken in 0.8 mL of dry methanol and 0.2 mL of *n*-butylamine added to it. To this was added a small amount of anhydrous sodium sulfate (5 mg) and a bead of Linde 4 Å molecular sieves. The mixture was kept under nitrogen at 4 °C overnight. The dark orange-red solution was filtered through a small Buchner filter directly into a Schlenk type storage vessel in which it was carefully evaporated to dryness using high vacuum (10^{-3} Torr). The solid was redissolved in 1.0 mL of dry methanol and stored under nitrogen at −20 °C. Protonation of Schiff base (1.0×10^{-5} M) was carried out by addition of dry methanolic HCl under nitrogen at 25 °C. Schiff bases of chromophores **II** and **III** were similarly prepared and handled.

Formation of Azo−Protein Complexes BR−I, BR−II, and BR−III and Competitive Binding Studies. The azo chromophores **I**, **II**, and **III** in ethanol were incubated with the apomembrane (4.0×10^{-5} M) in an aqueous medium, pH 5 (1.0×10^{-2} M Tris·HCl buffer) at ambient temperature in dim red light. The azo chromophore concentrations were 9.0×10^{-4} M in all cases, and the final ethanol concentration in the protein suspension was less than 3% v/v. Formation of the respective azo−protein complexes was monitored by UV−vis absorption spectra over a period of hours. After 24 h, the azo−protein complexes formed were washed free of excess chromophore by centrifugation (50000*g*, 10 min). At the end of 24 h, each of the incubation mixtures was supplemented with all-*trans*-retinal and the increase of absorbance at 560 nm was measured. The percent displacement was determined by measuring the ratio of the absorbance at the UV−vis maximum for the respective BR analogue at 60 min (the time taken for maximum displacement by retinal) and expressed as a percentage.

Fluorescence Studies. BR−**I**, BR−**II**, and BR−**III** were washed three times with 1.0×10^{-2} M Tris·HCl (pH 5) buffer and centrifuged (25000*g*, 30 min) to remove excess chromophore. The pellet was resuspended in 1.0×10^{-2} M Tris·HCl (pH 5). Bacterioopsin (BOP) and BR were also suspended in Tris buffer (pH 5). The optical density of all three solutions was adjusted to 0.5 (A_{280} = 0.5) before recording the fluorescent emission for 280 nm excitation.

Light-Induced pH Change Studies. Suspensions of native BR and the modified proteins BR−**I**, BR−**II**, and BR−**III** in basal salt of high optical density (A = 0.8 at the wavelength of maximum absorbance in the visible region) were prepared with the pH adjusted to 5 by the addition of HCl. No buffers were used as they inhibit pH changes. These were sonicated for 3 min using a Branson 450 sonifer at 4 °C. This suspension (4 mL) was taken in a tube with an outer jacket circulating water to maintain the temperature at 25 °C. The samples were illuminated with a Philips tungsten−halogen lamp (230 V, 500 W). The distance between the sample and the light source was 12 cm. pH changes were monitored with the pH meter. The amounts of protons displaced as a function of time were monitored by recording the change in pH upon turning the illumination source on and upon switching it off.

Flash Photolysis Studies. Basal salt suspensions of purple membrane fragments (A_{570} = 0.8) and the BR analogues BR−**I**, BR−**II**, and BR−**III** (A = 0.8 at the wavelength of maximum absorbance in the visible region) in Tris·HCl buffer (pH 5) were taken in a quartz cell for recording the flash photolysis decay profiles. The flash photolysis profiles of the transient species were monitored between 400 and 420 nm.

Results and Discussion

pH Dependent UV−Vis Characteristics of Chromophores I, II, and III. As evident from Table 1, the azo chromophores **I**−**III** show pH dependent UV−vis behavior. This is explained in Scheme 2. At high pH, the chromophore remains unprotonated. As the pH decreases, the availability of protons increases, and at sufficiently low pH, one of the nitrogen atoms of the azo group (−N═N−) becomes protonated, giving rise to large red-shifted bands (e.g., 511, 517, and 511 nm for chromophores

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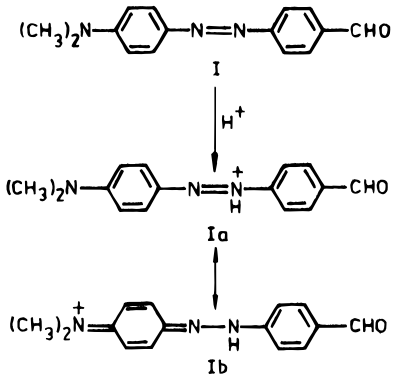
Table 1. UV−Vis Characteristics of Azo Chromophores **I**, **II**, and **III** in Methanol and at pH 3, 5, 7, and 9

	UV−Vis λ_{max} of chromophore (nm)[a]		
medium	**I**	**II**	**III**
pH 3[b]	511	517	511
pH 5[b]	405	388	395
pH 7[b]	412	387	393
pH 9[b]	416	385	392
methanol	449	461	450

[a] [Chromophore] = 1.0×10^{-5} M. [b] 1.0×10^{-2} M Tris·HCl buffer.

Scheme 2



I, **II**, and **III**, respectively). The more-red-shifted band (517 nm) of protonated chromophore **II** is due to the extended conjugation available in **II** which is not so in the case of **I**. However, for protonated chromophore **III**, the extended conjugation is prevented from playing a greater role in the stabilization of the positive charge due to the presence of a methyl group which can cause out-of-plane twisting of the bonds and disruption of extended conjugation. Thus, protonated **I** and protonated **III** show similar UV−vis absorption maxima at 511 nm despite dissimilarities in their chemical structures.

The protonation behavior of chromophores **I**−**III** throws light on the role of the availability or nonavailability of protons in a given environment and its importance in the wavelength-regulating property of the azo chromophore with changing pH. This may have some implications on the availability of protons at the active site environment of retinal proteins wherein the relative abundance of amino acid residues with ionizable H atoms in the vicinity of the retinylidene chromophore plays an important role in protonation/deprotonation and hence in wavelength regulation. Wavelength regulation could be effected by the partial (H-bonding type) or full protonation of the azo chromophore by the protein bound ionizable residues, resulting in species of the type shown in Scheme 2. This could be the cause for the appearance of the new red-shifted bands obtained by the interaction of the azo chromophores **I**, **II**, and **III** with bacterioopsin (BOP), resulting in the formation of BR−**I**, BR−**II**, and BR−**III**, respectively.

UV−Vis Characteristics of the *n*-Butylamine Schiff Bases of Chromophores I, II, and III and Their Protonation Behavior. The Schiff bases of azo chromophores **I**−**III** with *n*-butylamine were prepared, and their protonation behavior was studied to compare their UV−vis characteristics with those of the protein complexes BR−**I**, BR−**II**, and BR−**III** and to determine the respective opsin shifts.[10] The Schiff bases of azo chromophores **I**−**III** show interesting UV−vis characteristics on protonation with dry methanolic HCl (Table 2). The

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Table 2. UV−Vis Characteristics of Schiff Bases of **I**, **II**, and **III** with *n*-Butylamine and Their Protonated Salts

	UV−vis λ_{max} (nm)[a]			
chromophore	RCHO	SB[b]	PSB_1[c]	PSB_2[d]
I	449	436	465	520
II	461	446	504	541
III	450	434	484	542

[a] In methanol; [chromophore] = 1.0×10^{-5} M in each case. [b] SB = Schiff base. [SB] = 1.0×10^{-5} M. [c] PSB_1 = protonated Schiff base formed on protonation with 1:1 dry methanolic HCl (1.0×10^{-5} M HCl). [d] PSB_2 = protonated Schiff base formed on protonation with 1:2 dry methanolic HCl (2.0×10^{-5} M).

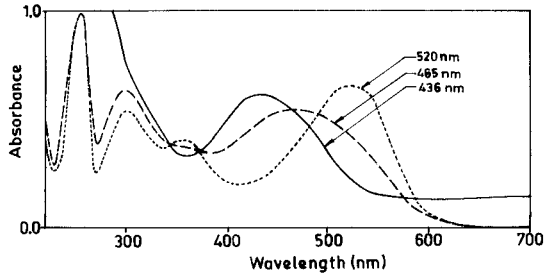
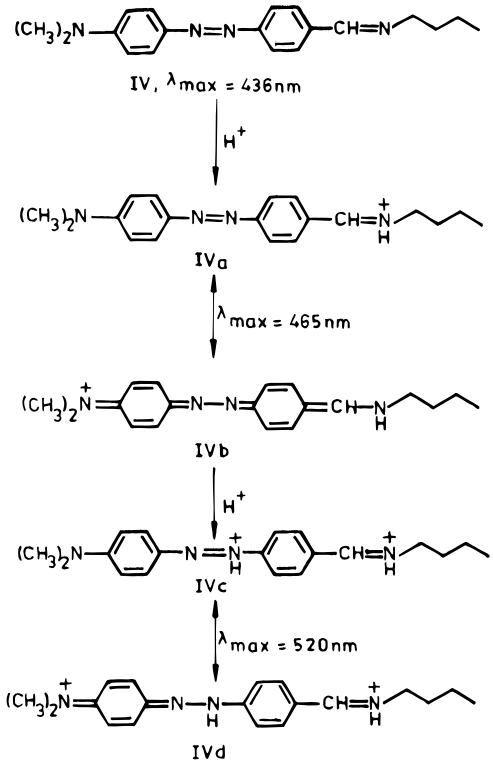


Figure 1. UV−vis characteristics of the *n*-butylamine Schiff base of azo chromophore **I**: [Schiff base] = 1.0×10^{-5} M, (—) 436 nm (in the absence of acid), (- - -) 465 nm (1:1 dry HCl, [HCl] = 1.0×10^{-5} M), (···) 520 nm (1:2 dry HCl, [HCl] = 2.0×10^{-5} M).

Scheme 3



absorption bands of the Schiff bases are found to be sensitive to the availability of protons in the medium. The Schiff base **IV** (Scheme 3) shows an absorption maximum at 436 nm and another intense band in the UV region (Figure 1). On addition of 1 equiv of methanolic HCl, the visible (436 nm) band shifts to 465 nm whereas a hypsochromic shift becomes apparent for the band in the UV region. This UV band resolves into two bands with absorption maxima at 254 and 295 nm. Addition of a larger amount of methanolic HCl (2 equiv) causes the visible band to further shift toward the red to 520 nm. Three

Table 3. UV−Vis Absorption Properties of Bacteriorhodopsin and Analogues Based on Chromophores **I**−**III**

	λ_{max} (nm)					
chromophore	RCHO[a]	RCHO[b]	SB[a]	PSB[a]	BR[b]	OS[c] (cm^{-1})
I	449	405	436	465	458	−329
II	461	388	446	504	597	3091
III	450	395	434	484	485	43
retinal	381	385	357	440	560	4870

[a] In methanol. SB = Schiff base of the respective chromophore with *n*-butylamine (1.0×10^{-5} M). PSB = protonated Schiff base. Protonation effected by 1:1 dry methanolic HCl. [b] In Tris·HCl buffer (1.0×10^{-2} M, pH 5). [c] Opsin shift.

bands at 254, 295, and 356 nm (minor band) in the UV region and isobestic points at 345 and 373 nm are also observed (Figure 1).

Protonation appears to take place in two stages; the −C═N− group is protonated first followed by the −N═N− group. This is consistent with the fact that while the pK_a of −C═N− is ∼7.5,[11] that of −N═N− is ∼3.0.[12] The stepwise protonation of the Schiff base of **I** is represented in Scheme 3. The nonprotonated Schiff base **IV** absorbs at 436 nm (Figure 1). On protonation with 1 equiv of acid, the imine nitrogen becomes protonated and the UV−vis absorption maximum shifts to 465 nm. The resonance-stabilized cation is shown by structures **IVa** and **IVb**. On addition of a further 1 equiv of acid, one of the azo nitrogens becomes protonated and the UV−vis absorption maximum shifts to 520 nm. The resultant dication is represented by the resonating structures **IVc** and **IVd**. Of these, structure **Vd** is expected to be the predominant contributing structure due to less repulsion between the positive charges. Moreover, aldehyde **I**, which does not contain the −C═N− group, on treatment with 1 equiv of dry methanolic HCl, shows a peak at 511 nm due to protonation of the −N═N− group. Therefore, the peak at 465 nm for the protonated Schiff base (**IVa**, **IVb**) could only be due to protonation of the imine (−C═N−) nitrogen atom.

The protonation behavior of the *n*-butylamine Schiff bases of **II** and **III** can be similarly rationalized. The UV−vis absorption properties of the *n*-butylamine Schiff bases of **I**, **II**, and **III** as well as their regulated, stepwise protonation with a distinct UV−vis absorption maximum for each protonated species afford a new approach to wavelength regulation in organic chromophores in general and azo-modified BR in particular.

Interaction of Azo Chromophores I, II, and III with BOP. Incubation of the ethanolic solutions of **I**, **II**, and **III** (9.0×10^{-4} M) with BOP (4.0×10^{-5} M) in a pH 5 buffer (Tris·HCl, 1.0×10^{-2} M) in the dark at 25 °C resulted in the formation of protein−chromophore complexes BR−**I**, BR−**II**, and BR−**III** derived from the azo chromophores **I**, **II**, and **III**, respectively. These were detected by following the change in UV−vis absorption (Table 3) with time. As typical examples, formation of the protein complexes BR−**I** and BR−**II** are shown in Figures 2 and 3, respectively. Chromophores **I**−**III** show pH dependent UV−vis absorption bands. At pH 5 (the pH at which binding studies were performed) chromophore **I** shows strong absorption bands in the range of 300−400 nm (λ_{max} = 289 and 405 nm). The band below 300 nm overlaps with the apoprotein absorption. After addition of compound **I** to the apoprotein at pH 5, the peak at 405 nm initially blue shifts to 395 nm, but over a period of time the band red shifts to 458 nm *via* a peak at 418 nm with an isobestic point at 408 nm (Figure 2). Binding of chromophore **II** with the apoprotein first results in a pigment

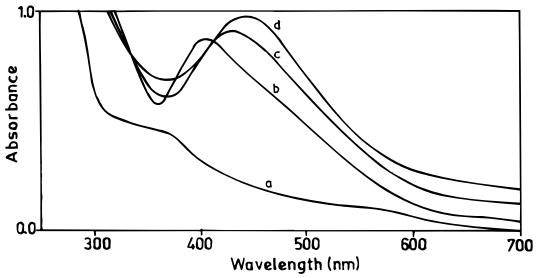


Figure 2. Formation of the azo−protein complex (progress with time) ([**I**] = 9.0×10^{-4} M, [BOP] = 4.0×10^{-4} M in 1.0×10^{-2} M Tris·HCl buffer): (a) before addition of azo chromophore **I**; (b) at 0 min, 395 nm; (c) after 30 min, 418 nm; (d) after 75 min, 458 nm.

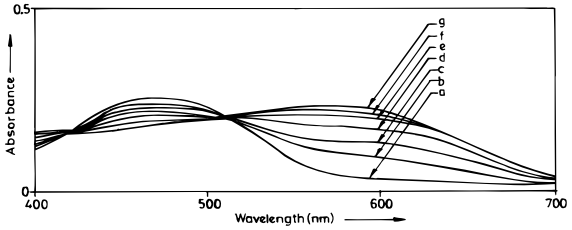


Figure 3. Formation of the 597 nm BR analogue from interaction of azo chromophore **II** ([**II**] = 9.0×10^{-4} M, [BOP] = 4.0×10^{-5} M in 1.0×10^{-2} M Tris·HCl buffer, pH 5, 25 °C). Spectra were recorded at different time intervals: (a) 0 min; (b) 5 min; (c) 10 min; (d) 20 min; (e) 30 min; (f) 40 min; (g) 50 min.

with UV−vis absorption at 487 nm which slowly shifts to 597 nm with isobestic points at 422 and 515 nm. Thus, analogue BR−**II** first forms a metastable pigment with an absorption maximum at 487 nm which becomes stabilized to a pigment with an absorption maximum at 597 nm over a period of time (Figure 3). Such stepwise binding of retinal analogues with BOP has been reported earlier.[2e] Chromophore **III** also binds with BOP, similar to chromophore **I**, with an initial UV−vis absorption maximum at 465 nm and a final absorption maximum at 485 nm obtained over a period of time, but without any clear cut isobestic point. It appears that, for all the cases, metastable pigments are formed before the final stable pigment is generated.

An estimation of half-lives and the rate of formation (*k*) of BR−**I**, BR−**II**, and BR−**III** (Table 4) indicates that chromophores **I**−**III** interact readily with BOP. Chromophores **I**, **II**, and **III** on reaction with BOP give new protein chromophores with absorption bands at 458, 597, and 485 nm, respectively. The rate constants (*k*) for the formation of BR−**I**, BR−**II**, and BR−**III** are 1.06×10^{-1}, 6.5×10^{-2}, and $<6.9 \times 10^{-2}$ min^{-1}, respectively, assuming first-order kinetics. Further, the largest bathochromic shifts are observed at pH 5. Studies at several other pH values (3, 7, and 9) did not result in significantly-red-shifted pigments though minor shifts of up to 10 nm were recorded for all the chromophores (**I**−**III**) incubated with BOP for up to 24 h at 25 °C. That such interaction occurs primarily at pH 5 is due to the pH sensitive nature of the chromophore, the particular protein conformation at that pH, and the interac-

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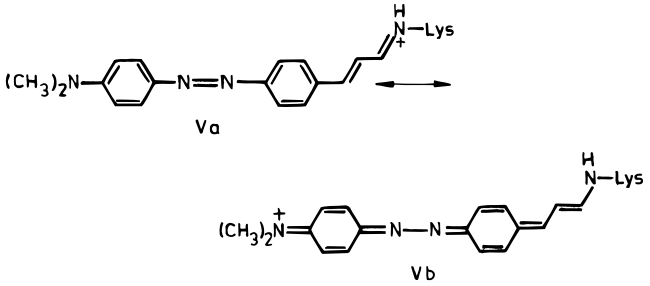
(12) *CRC Handbook of Chemistry and Physics*; Weast, R. C., Ed.; CRC Press Inc.: Boca Raton, FL, 1985; p D-159.

Table 4. Rate Constants for Formation of BR, BR−**I**, BR−**II**, and BR−**III** and Competitive Binding of Retinal with the Modified BR Pigments

protein	UV−vis maximum (nm)	rate constant[a] for formation of BR, k (min^{-1}) × 10^{-1}	$t_{1/2}$ (min)	percent[b,c] displacement of the azo chromophore by retinal (%)
BR−**I**	458	1.06	6.5	26.3
BR−**II**	597	0.65	10.6	15.4
BR−**III**	485	<0.69	<1.0	80.0
BR	560	1.50	4.6	

[a] First-order rate constants at 25 °C. In 1.0×10^{-2} M Tris·HCl, pH 5. [b] On addition of excess retinal in the dark. [c] Monitored for up to 24 h at 25 °C. Results obtained during the first 60 min do not change significantly at longer equilibration times.

Scheme 4



tions between the chromophores and the charge-carrying surface residues of the protein which are available to the chromophores.

The stability of the BR analogues, BR−**I**, BR−**II**, and BR−**III** was checked by addition of retinal to the respective BR analogue solutions and by monitoring the growth of UV−vis absorbance at 560 nm with time. For BR−**I**, there was 26.3% displacement of the chromophore by retinal, for BR−**II**, 15.4% displacement of the chromophore, while for BR−**III**, there was 80% displacement of the chromophore by retinal (Table 4). The competitive binding of retinal with BR−**I**, BR−**II**, and BR−**III** was monitored over a period of 24 h. Maximum displacement of the respective azo chromophores from the BR analogues by retinal occurred during the first 60 min. Insignificant displacement (not exceeding 5%) occurred during the remaining period of incubation; *i.e.*, the results obtained during the first 60 min do not change significantly at longer equilibration times.

Chromophores **I**−**III** showed considerable variation in their absorption properties and opsin shift data on interaction with BOP (Table 3). While the analogue BR−**I** showed a negative opsin shift of −329 cm^{-1}, analogue BR−**II** showed a large positive opsin shift of 3091 cm^{-1} and analogue BR−**III** showed only a small positive opsin shift of 43 cm^{-1}. The difference in the behavior of the three chromophores within the protein environment can be explained by considering the resonance structures **Va** and **Vb** (Scheme 4). The stabilization afforded by the extended conjugation and delocalization of the positive charge on the imine N atom of the protein complex of chromophore **II** results in the large red-shifted UV−vis maximum (597 nm as compared to 560 nm for native BR) and opsin shift (3091 cm^{-1}). The much lower UV−vis maximum (485 nm) and opsin shift (43 cm^{-1}) for the protein complex of **III** are due to the presence of the methyl group in the chain which offers steric hindrance to the proper recognition of chromophore **III** by the BOP binding site. This methyl group may also cause the out-of-plane twisting of the olefinic bond in the side chain of **III** within the restricted protein environment and consequent disruption of the extended conjugation and loss of stereoelectronic stability for the protein−chromophore complex. It is possible for the steric bulk of the α-methyl group to prevent the protein bound amino acid residues from approaching this region of the chromophore, leading to a lack of some vital interactions which enhance conjugation. Theoretical molecular mechanics calculations (PCMODEL) carried out on cinnamaldehyde and α-methylcinnamaldehyde show that while for α-methylcinnamaldehyde the olefinic side chain attached to the aromatic nucleus (as in compound **III**) is twisted by an angle of 64° with respect to the plane of the neighboring phenyl ring, for cinnamaldehyde, the chain (as in compound **II**) and the phenyl ring are found to lie on the same plane. These results are in agreement with theoretical and experimental studies carried out earlier on β-ionone and retinal which indicate a distorted *s-cis* conformation about the C6−C7 single bond with the torsional angle in the range 30−70°.[10,13] The protein−chromophore complex of **I** shows a still lower UV−vis absorption maximum (458 nm) as well as a negative opsin shift (−329 cm^{-1}). It is evident from the structure of **I** that the degree of stabilization attained for **II** and **III** is not possible in the case of **I** due to the absence of olefinic bonds in **I**, *i.e.*, the lack of longitudinal proximity of the chromophore to the apoprotein charges.

Table 5. Fluorescent Emission Data for BOP, BR, and Azo−Protein Complexes BR−**I**, BR−**II**, and BR−**III** (Excitation λ = 280 nm, 25 °C, 1.0×10^{-2} M Tris·HCl, pH 5)

fluorophore[a]	λ_{max} (nm)	K_{SV} (M^{-1}) × 10^6	fluorophore[a]	λ_{max} (nm)	K_{SV} (M^{-1}) × 10^6
BOP	339		BR−**II**	335	1.18[c]
BR	334	4.63[b]	BR−**III**	345	0.71[c]
BR−**I**	341	5.13[c]			

[a] [Fluorophore] = 1.5×10^{-6} M. [b] Obtained by measuring the quenching of protein fluorescence by retinal within bacteriorhodopsin in comparison to bacterioopsin. [c] Obtained by measuring the quenching of protein fluorescence by the chromophores **I**, **II**, and **III** within the chromophore−protein complex in comparison to bacterioopsin.

Fluorescence Behavior of BR−I, BR−II, and BR−III. The azo chromophores **I**−**III** are found to quench the fluorescent emission of the apoprotein of BR, BOP, when combined with it as azo−protein complexes BR−**I**, BR−**II**, and BR−**III** (Table 5). The fluorescent emission of BOP becomes quenched when it combines with retinal to form BR. Similar quenching is observed when BOP combines with azo chromophores **I**−**III**. The azo chromophores **I**−**III** also quench the fluorescent emission of tryptophan (Trp) in aqueous solutions.

It is known that the fluorescent emission of BR is quenched relative to BOP due to the interaction between the Trp residues lining the retinal pocket and the retinylidene chromophore. A similar interaction is envisaged to explain the observed quenching of the protein fluorescence by azo chromophores **I**−**III** in the azo−protein complexes BR−**I**, BR−**II**, and BR−**III** in comparison to BOP.

Light-Induced pH Change Activity of BR−I, BR−II, and BR−III. The light-induced pH change activity of the modified proteins BR−**I**, BR−**II**, and BR−**III** were studied and compared with that of native BR (Table 6). The studies were conducted at pH 5 as the modified BR analogues were formed and remain stable at this pH. It was found that while native BR is able to sustain repeated light-on/light-off cycles, only BR−**II** shows similar behavior. BR−**I** undergoes only one such cycle while BR−**III** does not exhibit any light-induced pH change activity.

This is because, while in the case of native BR, the protein is able to complete the photocycle ($BR_{570} \rightarrow J \rightarrow K \rightarrow L \rightarrow M \rightarrow ... \rightarrow BR_{570}$), such may not be the case with the modified proteins BR−**I** and BR−**III**. BR−**II** is functional and sustains

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Table 6. Functional Characteristics of Chromophore-Substituted Bacteriorhodopsins

protein	chromophore	light[a]-induced pH change	transient[b] absorption lifetime, τ (ms)
BR−**I**	**I**	+	1.5
BR−**II**	**II**	+	1.0
BR−**III**	**III**	−	
BR	retinal	+	7.0

[a] 1.0×10^{-2} M KCl−HCl, pH 5. [b] Excitation flash 400−700 nm, <100 μs.

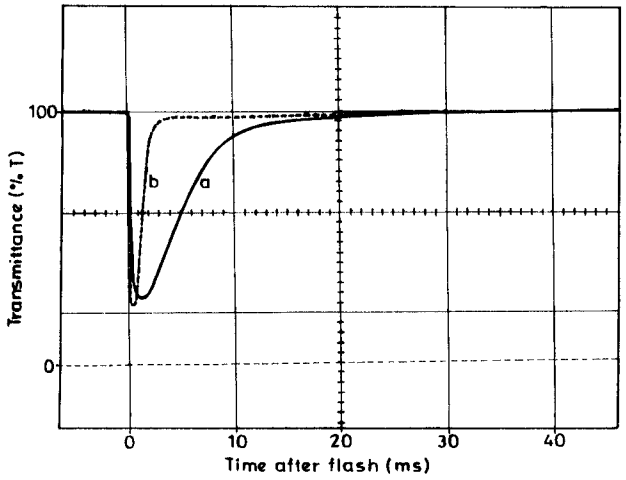

Figure 4. Flash photolysis traces for (a) native BR and (b) analogue BR−**II** with a maximum at 410 nm (*x* axis 1.0 ms per division; *Y* axis 0.5 V, 4.0% *T* per division) at 25 °C.

repeated light-on/light-off cycles due to the greater similarity between the chromophore of native BR and the chromophore of BR−**II**. This is not the case with BR−**I** and BR−**III**. Azo chromophore **I** can undergo isomerization in the presence of light inside the protein which may then change conformation, exposing fresh surface residues with ionizable protons. These protons are expelled by the protein, causing the observed pH changes. However, the protein may not be able to provide the energy to reverse the isomerization of azo chromophore **I**, and so the photocycle is not completed. Therefore, the pH change initially observed is not repeated. In the case of BR−**III** the protein environment is probably too restrictive for the molecular geometry of chromophore **III** to allow its photoisomerization and resultant light-induced pH change effects. However, more studies are required to fully understand this light-induced pH change activity of the analogues.

Flash Photolysis Studies. Modified BR containing azo chromophores **I**−**III** were flash photolyzed, and formation of transient species was recorded. Figure 4 shows the transient absorption for analogue BR−**II** and native BR. The transient lifetime (τ) is given by the time at $1/eI_{max}$ where I_{max} is the highest intensity of the signal. BR−**I** and BR−**II** produced transient species with lifetimes of 1.5 and 1.0 ms, respectively. BR−**III**, however, did not exhibit formation of such transient species. Under similar photolytic conditions, native BR produced a transient species with a lifetime of 7.0 ms at 410 nm. Thus, compared to the transient lifetime for native BR (7.0 ms), the modified BR analogues exhibit transient species with shorter lifetimes (Table 6). Formation of transient species in the case of BR−**I** and BR−**II** may be due to the photoisomerization of the protein bound azo chromophore (−N═N−). Formation of such transient species even for a chromophore which is devoid of a −C═C− bond, *e.g.*, BR−**I**, reinforces this assumption. However, contribution to the transient profile from −C═C− isomerization cannot be ruled out in the case of BR−**II**. BR−**III** does not show any detectable transient absorption which may be due to the steric hindrance of the methyl group which restricts the isomerization of **III** within BR−**III**.

Whether *trans*-azobenzene is indeed isomerized to *cis*-azobenzene was tested by steady state photolysis of chromophores **I** and **II**. Photolysis of the two chromophores **I** and **II** in methanol and in aqueous buffer (1.0×10^{-2} M Tris·HCl, pH 5) at their respective UV−vis absorption maxima using a monochromated source (450 W xenon arc lamp with a Spex 1681B Miniate-2 monochromator) does not result in any change in their UV−vis absorption maxima or absorbance. Thus, large spectral changes in the absorption spectra of the chromophores **I** and **II** are not observed under steady state photolysis conditions. However, this does not imply absence of *cis*−*trans* isomerization. It is known that extremely fast *Z* to *E* thermal recovery in protic solvents occurs when (*E*)-azobenzenes are photolyzed. Therefore, large spectral changes are not detected.[14,15] The chromophores studied by us have both electron-donating and electron-accepting groups; *i.e.*, they belong to the class of push−pull substituted azobenzenes. Steady state photolysis of these chromophores in methanol and in aqueous buffer does not result in observable spectral shifts probably due to hydrogen bond interactions between methanol/aqueous buffer and the chromophores **I** and **II**.

Moreover, in contrast to the usual time scale for *Z* to *E* thermal recovery of azobenzenes (*e.g.*, 100−500 μs for 4-(*N*,*N*-dimethylamino)-4′-nitro-1,1′-azodibenzene in various organic solvents at 30 °C),[16] the considerably different values for the azo-modified BR analogues indicates the influence of the surrounding protein environment on the photoisomerization of the chromophores **I**−**III**.

Nature of the Protein−Chromophore Interaction in BR−I, and BR−II, and BR−III in Terms of Longitudinal Restrictions of the Protein Binding Site and the External Point Charge Model. It is evident from the above discussion that there is strong interaction between the azo chromophores **I**, **II**, and **III** and the apoprotein. This is manifested by the altered physicochemical characteristics of BR−**I**, BR−**II**, and BR−**III** in comparison to native BR. An understanding of the factors involved for this altered behavior is necessary to explain the interactions occurring within these proteins. This may also throw new light on chromophore−protein interactions in native BR.

The chromophore binding site of BR imposes longitudinal restrictions on the retinylidene chromophore, which results in the highly regiospecific photoisomerization process.[17] For all-*trans*-retinal, the distance between the center of the trimethylcyclohexene ring and C-15 is 12.3 Å, whereas the 13-*cis*-retinal it is 11.2 Å. Similar distances calculated for azo chromophores **I**−**III** in the fully extended geometry are 11.0 Å for **I** and 13.8 Å for both **II** and **III**. Thus, the distances for **I**−**III** fall within the binding zone of BOP.

The role of point charges in causing the large bathochromic shift in native BR is much discussed.[10] A negative point charge is located at a distance of 3.5 Å above C-5 of the retinylidene chromophore of BR. A second negative point charge is located at a distance of 3.0 Å below the protonated Schiff base nitrogen and acts as the counterion. On the basis of the recently proposed

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three-dimensional model of BR, the charge above C-5 is due to Asp 96 and the counterion to the Schiff base is Asp 212.[18] The electronic and steric properties of the azo chromophores **I**, **II**, and **III** differ greatly from those of retinal, and the effect of the two protein bound point charges is expected to be very altered. While the trimethylcyclohexene ring of retinal is replaced by the phenyl ring in chromophores **I**−**III**, the presence of the −N═N− group provides additional sites for protonation/H bond interaction with charge-carrying protein bound residues. These interactions may place additional restrictions on the isomerization of the azo chromophore. The negative opsin shift of BR−**I** could have a partial contribution from the repulsive interaction between Arg 82 and the positive charge on the *N*,*N*-dimethylamino group of chromophore **I**, though in native BR the trimethylcyclohexene group is situated away from Arg 82. The effect of the positive charge on the *N*,*N*-dimethylamino group is offset by the availability of extended conjugation in the chromophores of BR−**II** and BR−**III**, resulting in net positive opsin shifts.

The residues Trp 86, Trp 138, Trp 182, and Trp 189 are present in the retinal pocket of the protein, and the quenching of fluorescent emission in BR−**I**, BR−**II**, and BR−**III** relative to BOP also points toward intimate interactions occurring between the respective azo chromophores and the protein bound residues.

Thus, careful evaluation of the available information on the azo-substituted BR−**I**, BR−**II**, and BR−**III** reveals several interesting facets of chromophore−protein interaction in BR.

Conclusion

The formation of BR−**I**, BR−**II**, and BR−**III** by the interaction of chromophores **I**, **II**, and **III**, respectively, with BOP indicates that the apoprotein can, under appropriate conditions, accommodate molecules in its binding site which have molecular geometries markedly different from those of retinal. It is also evident that the chromophores are effective pH sensitive probes for the inner hydrophobic domain of the protein.

The current investigations have defined a new direction for preparing novel BR-based pigments and also provide new insight into BOP−chromophore interactions.

Acknowledgment. The authors thank the reviewers for their valuable suggestions and the funding agencies (DST, DAE, CSIR, Government of India) for financial support.

JA954286X

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